SpA, ClfA and FnbpA genetic variations lead to Staphaurex test negative phenotypes in bovine mastitis Staphylococcus aureus isolates

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Abstract: Staphylococcus aureus encodes many proteins that act as virulence factors, leading to a variety of diseases, including mastitis in cows. Among these virulence factors, SpA, ClfA, ClfB, FnbA, and FnbB are important for the ability of S. aureus to adhere to and invade host cells as well as to evade host immune responses. The interaction between these S. aureus surface proteins and human immunoglobulin G and fibrinogen that are coupled to latex particles is utilized to induce latex agglutination reactions, which are used widely in diagnostic kits for confirmation of presumptive S. aureus isolates. In this study, the Staphaurex latex agglutination test was performed on a collection of confirmed bovine mastitis S. aureus isolates. Notably, 54% (43/79 isolates) of these isolates exhibited latex agglutination-negative phenotypes (Staphaurex-negative result). To gain insights into the reasons for the high frequency of Staphaurex-negative bovine mastitis S. aureus isolates, the spa, clfA, clfB, fnbA, and fnbB genes were examined. Specific genetic changes in spa, clfA, and fnbA, as well as a loss of fnbB, which may impair SpA, ClfA, FnbA, and FnbB functions in latex agglutination reactions, were detected in Staphaurex-negative S. aureus isolates. The genetic changes included a premature stop codon in the spa gene, leading to a truncated SpA protein that is unable to participate in S. aureus cell-mediated agglutination of latex particles. In addition, clfA and fnbA genetic polymorphisms were detected that were linked to ClfA and FnbA amino acid changes that may significantly reduce fibrinogen-binding activity. The genetic variations in these S. aureus isolates might also have implications for their bovine mastitis virulence capacity.

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SpA, ClfA, and FnbA Genetic Variations Lead to Staphaurex Test-Negative Phenotypes in Bovine Mastitis *Staphylococcus aureus* Isolates[▽]

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Staphylococcus aureus **encodes many proteins that act as virulence factors, leading to a variety of diseases, including mastitis in cows. Among these virulence factors, SpA, ClfA, ClfB, FnbA, and FnbB are important for the ability of *S. aureus* to adhere to and invade host cells as well as to evade host immune responses. The interaction between these *S. aureus* surface proteins and human immunoglobulin G and fibrinogen that are coupled to latex particles is utilized to induce latex agglutination reactions, which are used widely in diagnostic kits for confirmation of presumptive *S. aureus* isolates. In this study, the Staphaurex latex agglutination test was performed on a collection of confirmed bovine mastitis *S. aureus* isolates. Notably, 54% (43/79 isolates) of these isolates exhibited latex agglutination-negative phenotypes (Staphaurex-negative result). To gain insights into the reasons for the high frequency of Staphaurex-negative bovine mastitis *S. aureus* isolates, the *spa*, *clfA*, *clfB*, *fnbA*, and *fnbB* genes were examined. Specific genetic changes in *spa*, *clfA*, and *fnbA*, as well as a loss of *fnbB*, which may impair SpA, ClfA, FnbA, and FnbB functions in latex agglutination reactions, were detected in Staphaurex-negative *S. aureus* isolates. The genetic changes included a premature stop codon in the *spa* gene, leading to a truncated SpA protein that is unable to participate in *S. aureus* cell-mediated agglutination of latex particles. In addition, *clfA* and *fnbA* genetic polymorphisms were detected that were linked to ClfA and FnbA amino acid changes that may significantly reduce fibrinogen-binding activity. The genetic variations in these *S. aureus* isolates might also have implications for their bovine mastitis virulence capacity.**

Although *Staphylococcus aureus* strains are natural inhabitants of bovine skin and mucous epithelia, these bacteria can also infect bovine mammary glands, leading to mastitis. This process involves numerous cell surface virulence factors that promote cellular attachment and invasion as well as evasion of host immune responses and induction of toxic tissue reactions (24). The initial attachment of *S. aureus* to epithelial cells of the teat canal depends on the interaction of bacterial surface proteins, such as clumping factors A and B (ClfA and ClfB) and fibronectin-binding proteins A and B (FnbA and FnbB), with host fibrinogen and fibronectin proteins located in the basement membrane, around myoepithelial cells and fibroblasts (2, 21, 29, 37, 41). ClfB, apart from the iron-regulated surface determinant IsdA and the serine-aspartic acid repeat proteins SdrC and SdrD, was also shown to be important for the adhesion of *S. aureus* to squamous cells (9). Moreover, *S. aureus* is able to invade epithelial and endothelial cells as well as fibroblasts via binding of FnpA and -B to fibronectin, which provides a bridge to the $\alpha5\beta1$ integrin of host cells (34). The role of the fibronectin-binding proteins in adhesion and invasion of cells has also been shown for bovine mammary gland cells (25), but strain-associated differences were observed (7, 18).

Additionally, the *S. aureus* ClfA and -B and FnbA and -B proteins are involved in the evasion of host immune responses that may be directed against these pathogens. Based on their ability to bind fibrinogen on the *S. aureus* cell wall, they inhibit deposition of or access to opsonins to the pathogen (20). ClfA has been shown to impede macrophage phagocytosis (31) and to bind and activate the complement regulator factor I, thereby inactivating C3b, the central complement component (15). Other *S. aureus* surface proteins involved in host immune evasion are protein A (SpA) and IsdH (40). The immunoglobulin G (IgG)-binding SpA protein, in addition to B-cell superantigenic properties, has the capacity to coat the *S. aureus* cell surface with incorrectly oriented IgG molecules, thereby preventing phagocytosis as well as classical pathway complement fixation (3). Moreover, the ability of *S. aureus* to survive inside polymorphonuclear neutrophils contributes to immune evasion (13).

Adhesion and invasion levels, as well as the ability of intracellular *S. aureus* to induce apoptosis, depend on the bacterial growth phase and are subjected to regulation via the accessory gene regulator (*agr*) locus and the staphylococcal accessory regulator A (SarA) protein family (8, 25, 42). SarA upregulates the expression of *clfB* and *fnbA* and *-B* during the exponential growth phase, whereas *agr* downregulates *spa* and *fnbA* and *-B* expression in the postexponential growth phase (8). Although *clfA* is transcribed throughout the growth cycle, this gene is highly expressed in the postexponential growth phase (44). Additionally, *agr*- and *sarA*-dependent functions upregulate the expression of the four *S. aureus* hemolysins (alpha to delta) (8).

The Staphaurex test kit is a latex agglutination-based diagnostic tool that is used widely for the confirmation of putative *S. aureus* isolates. The function of this kit depends on the

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interaction of *S. aureus* surface-anchored proteins SpA, ClfA, ClfB, FnbA, and FnbB with human IgG and fibrinogen protein, which are bound on the surfaces of latex particles. These protein-protein interactions therefore mediate latex particle agglutination, which leads to visible clots in the case of a Staphaurex test positive (Staphaurex-positive) result, thereby confirming presumptive *S. aureus* organisms. As such, the specificity and sensitivity of this diagnostic test depend on the presence and proper function of the above-mentioned proteins on the surface of *S. aureus*. Some studies have reported low sensitivities and specificities for bovine *S. aureus* detection with latex agglutination-based tests, but the possible reasons for such observations have not yet been examined (35, 45).

The objectives of the present study were to characterize Staphaurex-negative and -positive bovine *S. aureus* isolates through evaluation of the genes corresponding to the Staphaurex test proteins, i.e., SpA, ClfA, ClfB, FnbA, and FnbB, as well as their expression. We could thereby assign potential reasons for the Staphaurex-negative results among our collection of bovine mastitis *S. aureus* isolates.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. A total of 79 presumptive *S. aureus* isolates were recovered from 78 cows coming from 61 herds in Switzerland. Bovine milk sample collection and isolation and identification of presumptive *S. aureus* organisms were done in accordance with International Dairy Federation (IDF) recommendations (20a). Subsequently, a previously described *S. aureus* species-specific 23S rRNA gene PCR analysis (36) was used to confirm the nature of the isolates. The isolates were stored at −70°C until used, at which time respective samples were streaked out onto sheep blood agar and incubated for 18 to 24 h at 37°C. Single *S. aureus* colonies were used for the analysis. Individual *S. aureus* colonies were inoculated into 10-ml cultures of brain heart infusion broth (BHI broth; Oxoid AG, Pratteln, Switzerland). The cultures were grown at 37°C in a shaking incubator set at 150 rpm until the stationary growth phase (18 to 24 h).

Phenotypic methods. A latex agglutination test was performed on *S. aureus* colonies following the instructions outlined in the Staphaurex test kit protocol (Remel, Oxoid AG, Pratteln, Switzerland). The production of hemolysins was determined on sheep blood agar, based on their interference with beta-hemolysin produced by the ATCC 25923 strain, in accordance with previously described classification criteria (26, 39). Based on these criteria, alpha (α) hemolysis is denoted as total hemolysis that is acuminated in the area of beta (β) hemolysis, which is partial hemolysis. Gamma (γ) hemolysis refers to hemolysis which is observed only on human or rabbit blood agar, while delta (δ) hemolysis denotes a CAMP-like total hemolysis in the area of beta hemolysis. The bovine *S. aureus* isolates were also tested for their egg yolk reaction on Baird-Parker agar (Oxoid AG, Pratteln, Switzerland) (4).

DNA purification. The *S. aureus* genomic DNA templates were extracted using a DNeasy blood and tissue kit (Qiagen AG, Hombrechtikon, Switzerland).

Amplification of genes. Regions of the *spa*, *clfA*, *clfB*, *fnbA*, and *fnbB* genes defined by the primers listed in Table 1 were amplified by conventional PCR. For each PCR, 10 ng of *S. aureus* genomic DNA template, a 500 nM concentration of each primer, and 25 μl of 2× GoTaq mixture (Promega AG, Dübendorf, Switzerland) were used in a 50-μl reaction mix. Amplification was carried out in a Biometra thermocycler (T3000), and the following parameters were applied: denaturation for 3 min at 95°C; 40 cycles of 15 s at 95°C, 30 s at 56°C, and 30 s (*spa*, *clfA*, and *clfB*), 60 s (*fnbB*), or 80 s (*fnbA*) at 72°C; and 5 min of extension at 72°C.

***spa* and *agr* typing.** The proline-rich repeat region of the *spa* gene was amplified from all *S. aureus* isolates (1), and the resulting amplicon sequences were analyzed on the SpaServer website (http://www.spaserver.ridom.de/) to assign a *spa* type to each isolate examined (16). The clonal complexes of the *spa* types were determined with Ridom StaphType software (version 1.0; Ridom GmbH, Würzburg, Germany). The *agr* groups (alleles) were determined by multiplex PCR as described by Gilot et al. (12).

MLST. Multilocus sequence typing (MLST) was performed for a selected number of isolates as described by Enright et al. (11). MLST results were analyzed with eBurst software (version 3.0 [http://www.mlst.net]) and were assigned to clonal complexes.

Total RNA extraction. Stationary-phase *S. aureus* cultures were prepared by growing single colonies in 10 ml BHI broth for 18 h at 37°C in a shaking incubator (150 rpm). Respective stationary-phase cultures (1.5 ml) were mixed with 3 ml of RNAprotect bacterial reagent (Qiagen AG, Hombrechtikon, Switzerland) and incubated for 5 min at room temperature to stabilize the RNA expression profiles before total RNA was extracted. Samples were centrifuged (10 min at 5,000 × *g*), and the bacterial cells were resuspended in 500 μl of lysis buffer provided in a Qiagen RNeasy Mini kit (Qiagen AG, Hombrechtikon, Switzerland). The samples were mechanically disrupted and total RNA isolated from the lysates based on the RNeasy Plus Mini kit protocol. Total RNA was eluted from the column into 50 μl of RNase-free water, and the RNA yield was determined using a NanoDrop ND-1000 instrument (NanoDrop Instruments, DE).

cDNA synthesis. A total of 300 ng of total RNA from respective samples was converted into cDNA in 20-μl reaction mixtures, using a QuantiTect reverse transcription kit (Qiagen AG, Hombrechtikon, Switzerland). Potential residual DNA contamination in each total RNA sample was assessed by including a control reaction without the reverse transcriptase enzyme (no-RT control) during cDNA synthesis. A calibrator RNA sample derived from a Staphaurex-positive *S. aureus* strain (isolate 1908) was included in each experimental run. The cDNA synthesis mixtures were incubated for 30 min at 42°C and 3 min at 90°C and then cooled to 4°C.

Relative gene expression quantification. Quantification of *spa*, *clfA*, *clfB*, and *fnbA* gene expression was performed using a LightCycler 480 real-time PCR instrument (Roche Diagnostics [Schweiz] AG, Rotkreuz, Switzerland). The quantitative RT-PCR (qRT-PCR) primers listed in Table 1 were used in 10-μl PCR mixtures which included LightCycler 480 SYBR green I master mix and 3.75 ng of diluted (1:10) cDNA template. The target gene transcripts were quantified using LightCycler 480 Advanced relative quantification software. The *S. aureus* DNA *gyrB* gene (43) and the 16S rRNA gene (10) were used as reference genes following confirmation of their stable expression between the different *S. aureus* isolates examined (32). Genomic DNA-based standard curves were used to define target and reference gene PCR amplification efficiencies for the different *S. aureus* isolates. These PCR efficiency values were applied in the subsequent calculation of relative gene expression levels for each isolate. All *S. aureus* isolates were assayed as duplicates in each experimental run, and in each case, two independent biological assays were performed.

Analysis of genetic variations. The complete *spa*, *clfA*, and *fnbA* genes in selected Staphaurex-negative and -positive *S. aureus* isolates were amplified using the PCR primers listed in Table 1, and the sequences of the resulting amplification products were determined. Based on *spa*, *clfA*, and *fnbA* gene sequence differences detected in Staphaurex-negative *S. aureus* isolates, real-time PCR primers (Table 1) were designed to specifically detect these sequence polymorphisms. Such primers were subsequently applied in real-time PCR assays to examine the frequencies of these detected genetic changes within the rest of the bovine mastitis-derived *S. aureus* isolates. The sequenced Staphaurex-negative and -positive isolates were used as controls, and a no-template reaction mix was used as a negative control.

PCR and gel purification. The PCR products were purified directly from amplification reaction mixtures or after agarose gel separation and band excision, using a MinElute PCR purification or MinElute gel extraction kit (Qiagen AG, Hombrechtikon, Switzerland), respectively.

Protein extraction and SpA immunoblotting. *S. aureus* protein fractions were extracted from selected isolates and immunoblotted as described by C. Quiblier et al. (submitted for publication). In brief, cell fractions and supernatant were sampled from a stationary-phase BHI culture. Cell wall fractions from each isolate were obtained after digesting the cells with SMM buffer (0.5 M sucrose, 0.02 M maleate [pH 6.5], 0.02 M $MgCl_2$) containing lysostaphin, lysozyme, DNase, and phenylmethylsulfonyl fluoride (PMSF). After resuspending the protoplasts in membrane buffer and lysing them by alternating freezing in liquid nitrogen and thawing at 37°C, the cytoplasmic fractions were collected. The membrane pellets were solubilized overnight at 4°C in solubilization buffer.

Twenty micrograms of protein from each isolate was separated in SDS-7.5% polyacrylamide gels and blotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore AG, Zug, Switzerland). After blocking of the membrane, it was incubated with goat anti-human IgA conjugated with horseradish peroxidase (HRP), diluted 1:2,000 in milk powder–phosphate-buffered saline (PBS) and Tween 20 (AppliChem GmbH, Darmstadt, Germany). HRP was detected with SuperSignal West Pico chemiluminescent substrate (ThermoFisherScientific, Lausanne, Switzerland).

TABLE 1. Oligonucleotide primers used in this study[i]

Target gene	Nucleotide sequence (5′–3′)	Reference or source
Primers for gene detection by conventional PCR		
spa	CAG CAG TAG TGC CGT TTG CTT	This study
	GAC GAT CCT TCA GTG AGC AAA G	This study
clfA	CGC CGG TAA CTG GTG AAG CT	This study
	TGC TCT CAT TCT AGG CGC ACT T	This study
clfB	ATG ATC TTG CTT GCG TT	This study
	CCG ATT CAA GAG TTA CAC C	This study
fnbA	GCG GAG ATC AAA GAC AAG TA[a]	5
	CAC CAT CTA TAG CTG TGT GG[a]	5
fnbB	GGA GAA GGA ATT AAG GCG[b,c]	5
	GCC GTC GCC TTG AGC GT[b]	5
	TSA CAG ARS TTA ACG ACT G[c]	This study
Primers for entire gene amplification by conventional PCR		
spa	ATC AAC GTA TAT AAG TTA AAA TTG GTT TGG A	This study
	ATA TTT ATT TTA TAA GTT GTA AAA CTT ACC TTT AAA TTT AAT T	This study
clfA	CAT TAA CAG AGA TTA AAT ATA TCT TTA AAG GGT	This study
	GGA AAA TCC AAG TAA AAA AGC CAC CT	This study
fnbA	TTT AAA AAC CGA ACA ATA TAG ACT TGC	This study
	GAA ATT TGA TTT AAT HTA AAA AAA CAG GYT T	This study
Primers for gene expression by qRT-PCR[d]		
spa	TTT GTC AGC AGT AGT GCC	This study
	ACG ATC CTT CAG TGA GC	This study
clfA	AGA AAC GCC GGT AAC T	This study
	CTC TCA TTC TAG GCG CAC	This study
clfB	GCG CAT TGG AAA TCG T	This study
	AGA GCC AGC TTC AAC A	This study
gyrB	GTC GAA GGG GAC TCT G	This study
	GCT CCA TCC ACA TCG G	This study
16S rRNA	TGT CGT GAG ATG TTG GG	This study
	CGA TTC CAG CTT CAT GT	This study
Primers for detection of sequence polymorphism by real-time PCR		
spa[e]	GAG CAT CGT TTA GCT TTT TAG CTT **A**[h]	This study
	TTC ATT CAA AGT CTT AAA GAC GA	This study
clfA[f]	AGA AAC GCC GGT AAC T	This study
	ACA TAG TCT GTA AAA GTA TAA ATA ACA TTA CCA **AA**[h]	This study
fnbA[g]	GTG AAT GGT TCG ATT GAG AC	This study
	CTT TAT ATT TGT TCT TAT CTG A**T**G **C**GT CT**A** **A**[h]	This study

[a] Modified from reference 5 in order to increase the melting temperature.
[b] Used for Staphaurex-negative isolates and for Staphaurex-positive isolates of *spa* types t267 and t524.
[c] Used for Staphaurex-positive isolates apart from those of *spa* types t267 and t524.
[d] Primers for qRT-PCR were designed using LightCycler probe design software (Roche Diagnostics [Schweiz] AG, Rotkreuz, Switzerland).
[e] Specific for premature stop codon in *spa* for Staphaurex-negative isolates.
[f] Specific for sequence polymorphism in EF-hand motif of *clfA* for Staphaurex-negative isolates.
[g] Specific for sequence polymorphism in *fnbA* for Staphaurex-negative isolates.
[h] Bold letters mark sequence polymorphisms at the following positions: for *spa*, 979; for *clfA*, 931 and 932; and for *fnbA*, 1246, 1247, and 1252 to 1254.
[i] All primers were produced by Microsynth AG, Balgach, Switzerland.

Statistical analysis. Box plots were drawn using the PASW Statistics 18.0 program (SPSS Schweiz AG, Zurich, Switzerland) to compare the gene expression ratios of the Staphaurex-negative and -positive *S. aureus* groups. The statistical significance of *spa*, *clfA*, and *clfB* gene expression differences between Staphaurex-negative and -positive *S. aureus* groups was determined based on repeated analysis of variance (ANOVA) measurements using the statistical program NCSS (NCSS, East Kaysville, UT). *P* values of <0.05 were regarded as significant.

RESULTS

Phenotypic characterization. Phenotypic characterization results for the 79 PCR-confirmed bovine mastitis-derived *S. aureus* isolates examined in this study are summarized in Table 2. Fifty-four percent (43/79 isolates) of the *S. aureus* isolates were Staphaurex negative. Since the expression levels of *spa*, *clfB*, *fnbA*, and *fnbB* are downregulated during the stationary phase, which corresponds to the growth state in colonies when the Staphaurex test is performed, we selected three Staphaurex-negative isolates and examined them at all growth stages. These isolates remained negative at all growth stages, indicating that their latex agglutination-negative phenotype was maintained at all stages of growth (data not shown). Furthermore, for all of these isolates, the negative latex agglutination phenotype was consistent for both blood agar-grown colonies and pelleted cells recovered from BHI broth cultures. All of the Staphaurex-negative isolates

TABLE 2. Phenotypic and genotypic characteristics of 79 bovine mastitis *S. aureus* isolates

No. of isolates in group	Staphaurex test result	*spa* type/CC[a]	No. of repeats in proline-rich repeat region of *spa*	*agr* type	MLST /CC (no. of typed isolates)[b]	Hemolysis type(s) (no. of isolates)	Egg yolk reaction(s) (no. of isolates)	Presence of change in gene		
								spa[c]	*clfA*[d]	*fnbA*[e]
11	+	t2953/CC8	10	I	ST8/CC8 (1)	Delta (6), alpha (4), none (1)	+	No	No	No
5	+	t267/CC80	10	I	ST352/CC97 (1)	Beta	+ (3)/− (2)	No	No	No
1	+	t024/CC8	9	I	ND	Delta	+	No	No	No
1	+	t5268/CC8	9	I	ND	Delta	+	No	No	No
1	+	t3802/CC8	9	I	ND	Delta	+	No	No	No
1	+	t034/CC398	9	I	ND	Alpha-beta	+	No	No	No
1	+	t2094/NA	8	I	ND	Beta	+	No	No	No
1	+	t7008/NA	7	I	ND	Alpha-beta	−	No	No	No
1	+	t045/CC5	7	II	ND	Alpha	+	No	No	No
2	+	t7007/NA	6	II	ST479/CC5 (1)	Alpha-beta	−	No	No	No
3	+	t7013/NA	5	II	ST479/CC5 (1)	Alpha-beta	+ (2)/− (1)	No	No	No
1	+	t1403/CC133	3	I	ST133/CC133 (1)	Alpha	−	No	No	No
7	+	t524/CC97	2	I	ST71/CC97 (2)	Beta	−	No	No	No
43	−	t529/CC151	2	II	ST504/CC705 (2)	Alpha-beta	−	Yes	Yes	Yes

[a] NA, not assignable.
[b] ND, not determined.
[c] Premature stop codon in *spa*, detected by sequencing or real-time PCR.
[d] Sequence polymorphism in EF-hand motif of *clfA*, detected by sequencing or real-time PCR.
[e] Sequence polymorphism at base positions 1246, 1247, and 1252 to 1254 in *fnbA*, detected by sequencing or real-time PCR.

exhibited alpha-beta hemolysis as well as egg yolk-negative phenotypes on Baird-Parker agar.

Genetic analysis. All bovine *S. aureus* isolates, including those that were negative for latex agglutination, were found to be positive for the *spa*, *clfA*, *clfB*, and *fnbA* genes when tested by PCR. In addition, *spa* gene amplification gave rise to eight PCR product band sizes, indicating the polymorphic nature of the variable proline-rich repeat region within *spa* genes across the *S. aureus* isolate collection examined (data not shown). Notably, all of the Staphaurex-negative isolates gave the same amplicon size, indicating that they harbored *spa* genes with similar variable repeat region lengths. This observation was subsequently confirmed by *spa* typing, which demonstrated that all Staphaurex-negative isolates were of the t529 *spa* type (Table 2). All of the Staphaurex-negative isolates were negative for *fnbB*, whereas all but five Staphaurex-positive isolates were found to be *fnbB* positive by PCR (data not shown). Next, the isolates were further typed based on *agr* genetic polymorphisms, since *agr* constitutes one of the global virulence gene expression regulators that control *spa* and *fnbA* and -*B* expression, which might thus also influence latex agglutination phenotypes. All of the Staphaurex-negative isolates were of *agr*

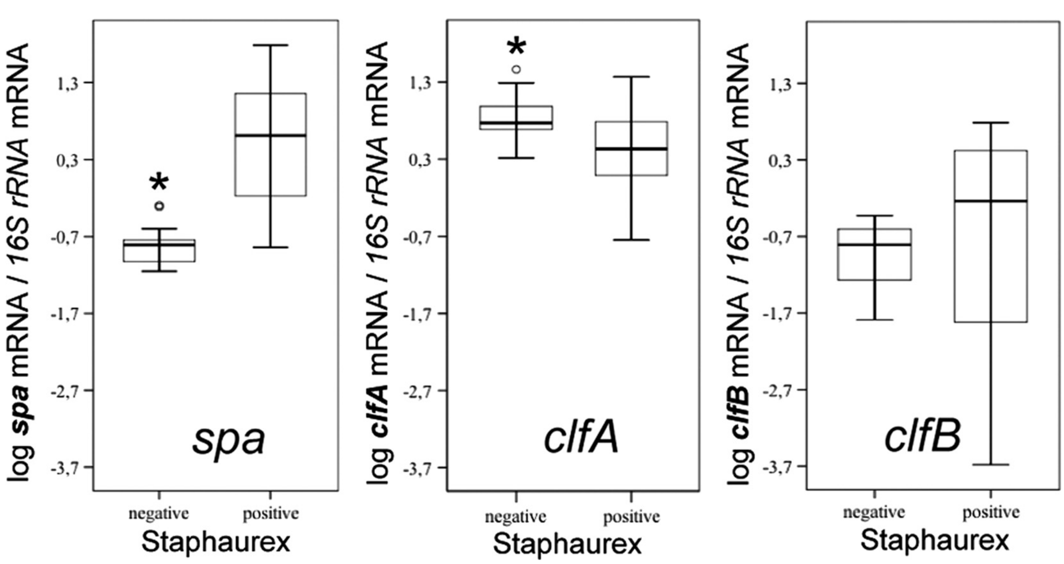

FIG. 1. Box plots showing the *spa*, *clfB*, and *clfA* relative gene expression levels in Staphaurex-negative ($n = 17$) and -positive ($n = 11$) bovine mastitis *S. aureus* isolates. Staphaurex-negative isolates showed significantly (*, $P < 0.05$) lower expression of *spa* but significantly higher expression of *clfA* than did Staphaurex-positive isolates. No significant differences were detected in *clfB* relative gene expression levels between the two groups of *S. aureus* isolates.

TABLE 3. Sequence polymorphisms found in sequenced *spa*, *clfA*, and *fnbA* genes

Relevant gene and *spa* type or reference strain (no. of isolates)	Staphaurex test result	Relevant sequence[i]	Position[a]	Reference
spa				
t529 (5)	–	CAAAGAAATTTTAGCA**T**AA	979	This study
All other types[b] (11)	+	CAAAGAAATTTTAGCAGAA	982–984	This study
clfA				
Newman[c]		GAT GGT DS D G NVIYTFTD	934–939[d] 310–321	30
t529 (4)	–	**TT**T GGT DS F G NVIYTFTD	931–932 311	This study
t7007 (1)/t7013 (2)	+	GAT G*A*T DS D D NVIYTFTD	938 313	This study
t2953 (1)/t267 (1)	+	GAT GGT DS D G NVIYTFTD	934–939 312–313	This study
fnbA				
NCTC 8325-4[e]		GCA AAT ACG A N T	1243–1251[f] 415–417 (I)[g]	23
t529 (3)	–	**TT**A GAC **GCA** L D A	1246/1247/1252/1254 416/418 (VI)[g]	This study
t524 (1)	+	GCA AAC A*T*A A N I	671–672[h] 224	This study
t2953 (1)	+	GCA AAT ACG A N T	664–672[h] 222–224	This study

[a] Nucleotide or amino acid sequence-based regions of interest in *S. aureus* reference strains and bovine mastitis isolates. Position numberings in some cases differ due to gene length differences in the respective *S. aureus* reference strains and examined isolates.
[b] The other sequenced *spa* genes of our Staphaurex-positive isolates of *spa* types t2953, t267, t7007, t7013, t1403, and t524.
[c] Available in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under GenBank accession no. Z18852.1 (October 2010) (28) (bases 302 to 3103).
[d] Positions based on the *clfA* part of sequence Z18852.1 only (bases 302 to 3103 = positions 1 to 2802).
[e] Available under GenBank accession no. J04151.1 (October 2010) (33) (bases 118 to 3174).
[f] Positions based on the *fnbA* part of sequence J04151.1 only (bases 118 to 3174 = positions 1 to 3057).
[g] Positions 415 to 417 in FnbpA isoform I (27) are positions 416 to 418 in isotype VI.
[h] N2,N3 (fibrinogen-binding) domain only was sequenced, and positions are based on the N2,N3 domain in NCTC 8325-4 (bases 697 to 1650 = positions 1 to 954).
[i] Bold letters indicate nucleotide base changes in Staphaurex-negative *S. aureus* isolates, underlining indicates condon or amino acid changes detected in both Staphaurex-positive and -negative isolates compared to the reference *S. aureus* strains, and letters in italic indicate nucleotide base changes in Staphaurex-positive *S. aureus* isolates.

type II (Table 2). In contrast, the majority (84%) of Staphaurex-positive isolates were of *agr* type I, apart from the isolates harboring *spa* type t045, t7007, or t7013, which were of *agr* type II. Nine isolate representatives were selected from this *S. aureus* collection and subjected to further analysis by MLST. These results are also presented in Table 2.

***spa*, *clfA*, *clfB*, and *fnbA* gene expression analysis.** The levels of *spa*, *clfA*, *clfB*, and *fnbA* gene transcripts in stationary-phase cells of selected Staphaurex-negative ($n = 17$) and -positive ($n = 11$) isolates were determined by real-time qRT-PCR. Box plots summarizing the relative *spa*, *clfA*, and *clfB* gene expression levels across the Staphaurex-negative and -positive groups are presented in Fig. 1. As shown, we found that *spa* transcript levels across the Staphaurex-negative group were significantly ($P < 0.05$) below those of the Staphaurex-positive group. The expression of *clfA*, on the other hand, was significantly ($P < 0.05$) higher for the Staphaurex-negative group than for the Staphaurex-positive group. In contrast, there were no significant differences ($P > 0.05$) in both *clfB* and *fnbA* gene expression levels (data not shown) between these two groups.

Evaluation of *spa*, *clfA*, and *fnbA* gene sequences. The *spa*, *clfA*, and *fnbA* gene sequences were determined in a selection of Staphaurex-negative and -positive isolates (Table 3). The *spa* gene sequences from all sequenced Staphaurex-negative isolates featured a G-to-T base change at position 979, which leads to a premature stop codon after 326 amino acids in the translated SpA protein sequence. In contrast, none of the sequenced Staphaurex-positive isolates had similar changes in their respective *spa* sequences.

The ClfA lengths based on the determined full-length *clfA* gene sequences ranged from 854 to 934 amino acids in the different *S. aureus* isolates. All sequenced Staphaurex-negative isolates displayed GA-to-TT nucleotide differences at positions 931 and 932 compared to the Newman reference strain sequence (30). This change in the *clfA* sequence resulted in a D311F amino acid change in the ClfA protein sequence.

The major genetic variations found in the Staphaurex-negative isolate *fnbA* sequence compared to the NCTC 8325-4 reference strain (23) included an A416L amino acid change due to GCA-to-TTA base changes at positions 1246 to 1248, as

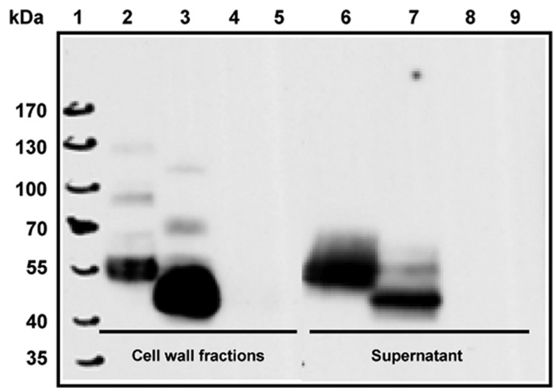

FIG. 2. Western blot analysis of cell wall fractions and supernatants of Staphaurex-positive and -negative bovine mastitis *S. aureus* isolates. Proteins derived from the cell wall fractions of stationary-phase BHI cultures and their concentrated supernatants were resolved by SDS-PAGE. The proteins were analyzed by Western immunoblotting using an HRP-conjugated goat anti-human IgA. Lane 1, molecular size marker; lanes 2 and 6, Staphaurex-positive *S. aureus* isolate 1887; lanes 3 and 7, Staphaurex-positive *S. aureus* isolate 1917; lanes 4 and 8, Staphaurex-negative *S. aureus* isolate 1910; lanes 5 and 9, Staphaurex-negative *S. aureus* isolate 2188. The expected SpA protein sizes are 57.2 (520 aa) and 50.1 (456 aa) kDa for Staphaurex-positive isolates 1887 and 1917, respectively. In the Staphaurex-negative isolates 1910 and 2188, the expected truncated SpA protein size would be 35.8 kDa (326 aa).

well as a T418A amino acid change due to ACG-to-GCA base changes at positions 1252 to 1254.

All of these genetic variations in *spa*, *clfA*, and *fnbA* were confirmed to be present in the rest of the Staphaurex-negative *S. aureus* isolates (Table 2) by use of a real-time PCR and primer sets that were specifically designed to detect these sequence variations. In contrast, none of these genetic changes were detected in any of the Staphaurex-positive isolates examined in this study.

SpA detection by immunoblotting. The expression of SpA was examined at the protein level in four selected isolates, representing two Staphaurex-positive and two Staphaurex-negative isolates. To achieve this, supernatant, cell wall, cytoplasmic, and membrane protein fractions as well as total protein were extracted from two Staphaurex-positive (*spa* types t2953 and t524) and two Staphaurex-negative isolates. As an example, immunoblotting results are shown in Fig. 2, where goat anti-human IgA-binding activity was assessed in the cell wall fractions and culture supernatants. As shown for both Staphaurex-positive isolates, bands corresponding to the expected size of translated full SpA (55 kDa for *spa* type t2953 and 48 kDa for *spa* type t524) were detected. In contrast, there was no corresponding SpA-associated human IgG-binding activity detected in samples derived from the two Staphaurex-negative isolates.

DISCUSSION

The latex agglutination-based Staphaurex test kit is reported to have a specificity and sensitivity of 99.5% and 99.8%, respectively, when applied to *S. aureus* isolates of human origin, according to the information provided by the kit manufacturer. However, in the present study, a high frequency (54%) of Staphaurex-negative results was found among PCR-confirmed *S. aureus* isolates of bovine mastitis origin. Since Atkins et al. (3) did not detect any host species-specific selection for immunoglobulin binding in SpA, we hypothesized that there might be differences in the execution of protein-protein interactions (SpA with human IgG and/or ClfA, ClfB, FnbA, or FnbB with human fibrinogen) between the latex agglutination-negative and -positive *S. aureus* isolates examined in our study. Possible reasons for Staphaurex-negative results might include loss or inadequate expression of one or more Staphaurex-targeted *S. aureus* genes (*spa*, *clfA*, *clfB*, *fnbA*, and *fnbB*). Moreover, natural genetic variability among isolates might result in adhesion proteins that are impaired in interaction with the human IgG and fibrinogen targets which are coupled to the latex particle surfaces in the Staphaurex test kit.

All of the latex agglutination-negative *S. aureus* isolates of bovine mastitis origin examined in our study possessed the *spa*, *clfA*, *clfB*, and *fnbA* genes. However, some key genetic differences were detected in the *spa*, *clfA*, and *fnbA* gene sequences of Staphaurex-negative isolates. These differences lead to truncated SpA proteins as well as to ClfA and FnbA proteins that may have lower fibrinogen-binding affinities.

All Staphaurex-negative *S. aureus* isolates examined displayed a genetic change in *spa* that leads to a premature stop codon and a truncated SpA protein. This premature stop codon is located within the fifth IgG-binding domain of SpA, which leaves the proline-rich repeat, cell wall, and membrane-spanning regions of the protein untranslated. As a result, such SpA proteins might not be anchored to the *S. aureus* cell surface because they lack the sortase motif (LPETG) and therefore would not be able induce latex agglutination reactions in the Staphaurex test. Moreover, based on Western immunoblotting, the truncated SpA protein, if expressed in Staphaurex-negative *S. aureus* isolates, seems to have a significantly impaired immunoglobulin-binding affinity. Interestingly, the same genetic change leading to SpA truncation is present within the *spa* gene of ET3-1 (RF122; GenBank accession no. AJ938182.1), the only *S. aureus* strain of bovine origin to date whose genome has been sequenced completely (19).

In addition, there were *clfA* genetic polymorphisms detected in all of the Staphaurex-negative isolates that induced an amino acid change at position 311, within the ClfA Ca^{2+}-binding EF-hand motif. Similar mutations have previously been found to reduce fibrinogen-binding affinity when artificially introduced into the *S. aureus* Newman strain ClfA protein (30). The amino acid at position 311 is a cation-coordinating residue, and only acidic (D) or polar (N/S) amino acids are predicted to be functionally acceptable residues in this position. O'Connell et al. (30) showed that while a D310A mutation had no effect on fibrinogen binding, a D310A and D312A double mutation induced a 3-fold reduction in the ClfA fibrinogen-binding capacity of the Newman strain. We therefore assumed that the D311F (D312A in strain Newman) change observed in our Staphaurex-negative isolates might also lead to reduced ClfA fibrinogen-binding activity because phenylalanine, like alanine, is a nonpolar amino acid, which is a functionally nonacceptable residue in this position. Herron-Olson et al. (19) showed that *clfA* of ET3-1 contains a premature stop codon due to an adenine (A) deletion between positions 36 and 42 (with ATG as the start codon), which results in a translational frameshift, leading to several premature stop codons in the translated protein. To examine such a possibility within the *clfA* genes of the *S. aureus* isolates in the present study, some Staphaurex-negative and -positive isolates as well as the ET3-1 strain were sequenced. The resequencing of *clfA* in ET3-1 confirmed the previously described A deletion, but none of the sequenced *clfA* genes of our Staphaurex-positive and -negative isolates displayed any base deletion that would result in such a translational frameshift.

Moreover, all Staphaurex-negative isolates examined in our study harbored A416L and T418A amino acid changes in FnbA. Interestingly, amino acid changes at the same positions, introduced by mutagenesis into the NCTC 8325-4 reference strain, were shown to significantly reduce the fibrinogen-binding capacity of FnbA (23). The same *fnbA* genetic polymorphisms as those in our Staphaurex-negative isolates were also observed in the sequenced ET3-1 strain (19). Seven FnbA isotypes have been defined (27). The FnbA sequences determined for the Staphaurex-negative isolates of our collection were all of isotype VI. These isolates have one amino acid more than the protein of NCTC 8325-4 (isotype I), preceding the amino acid changes mentioned above. Therefore, amino acid positions 416 to 418 in our Staphaurex-negative isolates correspond to positions 415 to 417 in NCTC 8325-4. An additional N417D FnbA amino acid change was found in all of the sequenced Staphaurex-negative isolates, but its potential impact on fibrinogen binding is presently unknown.

The contribution of *S. aureus* FnbB to the latex agglutination reaction has been demonstrated previously. By expressing FnbpB of strain 8325-4 of *Staphylococcus carnosus*, Grundmeier et al. (14) showed that FnbB alone was sufficient to cause latex agglutination. Meanwhile, all Staphaurex-negative isolates, including ET3-1, failed to give a PCR product when amplified with a set of primers targeting the *fnbB* gene, as previously described (5). Although it cannot be ruled out completely that the primer set used might fail to amplify the different FnbpB isotypes due to possible sequence polymorphisms in primer binding regions (6), we presume that similar to ET3-1, our Staphaurex-negative isolates also lack the *fnbB* gene.

It is interesting that the latex agglutination-negative *S. aureus* isolates in our study share various phenotypic and genetic similarities with the ET3-1 strain, which is a member of one of the most abundant *S. aureus* lineages associated with intramammary infection of cows from diverse localities (22). Specifically, we found that compared to the Staphaurex-negative bovine mastitis-derived *S. aureus* isolates of our study, the ET3-1 strain is also Staphaurex negative, shows alpha-beta hemolysis, is of *spa* type t529 and *agr* type II, and displays the same key genetic changes in *spa* and *fnbA*. Additionally, ET3-1 belongs to ST151, whereas our Staphaurex-negative isolates belong to ST504, sharing five of seven loci with ST151. Both MLST types are grouped into the same 705 clonal complex (17).

Another remarkable observation was that some of the *clfA* and *fnbA* genetic changes that were originally discovered through artificial mutagenesis in laboratory strains to reduce the fibrinogen-binding capacities of ClfA and FnbA, respectively, were also found to occur naturally in latex agglutination-negative *S. aureus* isolates, as shown in the present study.

Meanwhile, it was interesting that all Staphaurex-negative *S. aureus* isolates were of *agr* type II, while the majority (83%) of Staphaurex-positive isolates were of *agr* type I. The dominance of *agr* types I and II in bovine *S. aureus* isolates was also shown previously by Gilot et al. (12). Buzzola et al. (7) demonstrated that *agr* type II *S. aureus* isolates displayed a significantly decreased level of internalization into MAC-T (bovine mammary epithelial cell line) cells *in vitro*. One plausible hypothesis would be that this might be linked to a higher degree of suppression of *spa* and *fnbA* and *-B* expression by *agr* type II than by *agr* type I isolates, but this remains to be investigated. Our gene expression results showed low *spa* expression levels in *agr* type II-harboring Staphaurex-negative isolates as well as in the few Staphaurex-positive isolates also harboring the *agr* type II sequence. An inverse relationship between alpha hemolysis and SpA, both of which are regulated by *agr*, was found by Takeuchi et al. (38) for bovine mastitis-derived *S. aureus* isolates. Similar observations were made in our current study, as all of the Staphaurex-negative isolates displayed alpha-beta hemolysis and little *spa* gene expression, while 54% of Staphaurex-positive isolates displayed only delta or beta hemolysis and relatively higher *spa* expression levels.

Despite the expression of *clfA* in stationary-phase cultures, Staphaurex-negative isolates were found in great numbers, indicating the possible impact of the amino acid change within the EF-hand motif of ClfA in fibrinogen binding. Meanwhile, our observation that *clfB* was lowly expressed in stationary-phase cultures (Fig. 1) parallels observations by Ní Eidhin et al. (29), who detected ClfB only on cells grown to early exponential phase. ClfB might no longer be present on the cell surfaces of most stationary-phase cells, thereby contributing less to fibrinogen binding during latex agglutinaton in Staphaurex test.

The reasons for latex agglutination-negative results previously mentioned in the literature include experimental differences, missing *S. aureus* surface proteins, and the use of nonhuman IgG (45). Our observations here suggest that, in the Staphaurex-negative *S. aureus* isolates examined, the SpA protein is truncated and not attached to the cell wall. Furthermore, this protein might not be expressed sufficiently in Staphaurex-negative isolates, based on our qRT-PCR results. Even if it is expressed at the protein level, the expressed form of this SpA protein might have poor immunoglobulin-binding activity, since it could not be detected by immunoblotting. Either way, the SpA protein in Staphaurex-negative isolates seems not able to participate in latex agglutination reactions. On the other hand, there were no significant differences in *clfB* and *fnbA* (data not shown) transcript levels between Staphaurex-positive and -negative isolates, while *clfA* transcripts were higher in Staphaurex-negative isolates. It still remains to be determined if these differences are reflected at the protein expression and functional levels. Protein sequence analysis suggests, however, that the ClfA and FnbA proteins in *S. aureus* isolates might be impeded from giving observable positive latex agglutination phenotypes due to their impaired binding to the fibrinogen molecules that are coupled to the latex particles in the Staphaurex kit.

The Staphaurex-positive isolates described in this study, on the other hand, constituted a more diverse group. As shown in Table 2, this category included isolates displaying alpha, beta, alpha-beta, or delta hemolysis and one nonhemolytic isolate, as well as isolates that were positive or negative for the egg yolk reaction. Moreover, there were 13 different *spa* types present, with at least 6 different MLST types and 2 *agr* types: 83% (30/36 isolates) of the isolates were of *agr* type I, and 17% (6/36 isolates) were of *agr* type II. Sequenced FnbA proteins (N2,N3 region) were of isotypes I (*spa* type t2953) and IV (*spa* type t524) (27). None of the Staphaurex-positive *S. aureus* isolates harbored any of the genetic changes observed in the Staphaurex-negative *S. aureus* isolates, apart from a few Staphaurex-positive isolates that failed to give a PCR band corresponding to *fnbB* amplification. As already mentioned, the failure to give *fnbB* amplicons might also be due to the fact that the primer set applied might not capture all FnbB isotypes.

As shown in the present study, the Staphaurex-negative phenotype observed in 54% of isolates might be linked to genetic changes affecting the interaction of SpA, ClfA, ClfB, and FnbpA with host IgG and fibrinogen, which might also have virulence implications for the respective *S. aureus* isolates. This study included five *S. aureus* isolates that were associated with mastitis herd problems. Interestingly, four of these isolates were Staphaurex positive, and only one was a Staphaurex-negative isolate. This Staphaurex-negative *S. aureus* isolate was detected with a Staphaurex-positive *S. aureus* isolate in the same herd. Thus, its independent role in this mastitis herd problem cannot be confirmed. Since SpA, ClfA, and FnbA and -B are important *S. aureus* virulence factors, their impaired functionality in Staphaurex-negative isolates may be presumed to lead to reduced virulence. However, such a hypothesis awaits further experimental confirmation. If it holds true, the Staphaurex test kit could potentially serve as an important diagnostic tool for rapid differentiation of more or less virulent *S. aureus* isolates in association with bovine mastitis.

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